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Mad2, Bub3, and Mps1 regulate chromosome segregation and mitotic synchrony in *Giardia intestinalis*, a binucleate protist lacking an anaphase-promoting complex

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ABSTRACT **The binucleate pathogen *Giardia intestinalis* is a highly divergent eukaryote with a semiopen mitosis, lacking an anaphase-promoting complex/cyclosome (APC/C) and many of the mitotic checkpoint complex (MCC) proteins. However, *Giardia* has some MCC components (Bub3, Mad2, and Mps1) and proteins from the cohesin system (Smc1 and Smc3). Mad2 localizes to the cytoplasm, but Bub3 and Mps1 are either located on chromosomes or in the cytoplasm, depending on the cell cycle stage. Depletion of Bub3, Mad2, or Mps1 resulted in a lowered mitotic index, errors in chromosome segregation (including lagging chromosomes), and abnormalities in spindle morphology. During interphase, MCC knockdown cells have an abnormal number of nuclei, either one nucleus usually on the left-hand side of the cell or two nuclei with one mislocalized. These results suggest that the minimal set of MCC proteins in *Giardia* play a major role in regulating many aspects of mitosis, including chromosome segregation, coordination of mitosis between the two nuclei, and subsequent nuclear positioning. The critical importance of MCC proteins in an organism that lacks their canonical target, the APC/C, suggests a broader role for these proteins and hints at new pathways to be discovered.**

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INTRODUCTION

Mitotic chromosome segregation is a highly regulated process that ensures the proper distribution of genetic material between daughter cells to avoid aneuploidy. Eukaryotic cells have evolved molecular mechanisms to assure that chromosome segregation is accurate, including an evolutionarily conserved checkpoint pathway known as the spindle assembly checkpoint (SAC) or mitotic checkpoint (MC) that is found in metazoans, yeasts, and plants (reviewed in Vleugel *et al.*, 2012; Lara-Gonzalez *et al.*, 2012). After kinetochores assemble at the centromere, they are competent to bind to microtubules from either spindle pole in any configuration. For proper segregation, however, sister chromatid kinetochores must be attached to microtubules from opposite spindle poles so that when sister chromatid cohesion is released at anaphase, one sister chromatid moves to one pole and the other to the opposite pole (Vitre and Cleveland, 2012). The MC halts mitotic progression in metaphase until the kinetochore of each sister chromatid is attached to just one of the two sets of microtubules radiating from poles of the spindle. During this time, the two sister chromatids are held together by a molecular ring, the cohesin complex, formed by the proteins Smc1, Smc3, Scc1, and Scc3 (reviewed in Remeseiro and Losada, 2013). Once all sister chromatids are properly attached to the mitotic spindle, the inhibitory signal generated at the kinetochore by the MC is turned off, the cohesin complex is destroyed by separase, and anaphase chromosome segregation begins.

At the core of this checkpoint system is the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase complex that regulates kinetochore function, sister chromatid cohesion (SCC),

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Abbreviations used: APC/C, anaphase-promoting complex/cyclosome; DAPI, 4′,6-diamidino-2-phenylindole; HA, hemagglutinin; HRP, horseradish peroxidase; MC, mitotic checkpoint; MCC, mitotic checkpoint complex; MTOC, microtubule-organizing center; SAC, spindle assembly checkpoint; SCC, sister chromatid cohesion; SPB, spindle pole body.

and mitotic progression by ubiquitinating specific cell cycle regulators to target them for degradation through the proteasome. Specifically, APC/C is able to tag securin, Aurora kinase, and cyclin B for degradation. Aurora kinase phosphorylates a variety of substrates required for chromosome segregation, including kinetochore components such as Ndc80 and the cohesion complex, altering their function or turnover. The degradation of securin activates the protease separase, which degrades the cohesin subunit Scc1 and releases sister chromatid cohesion, allowing chromosome separation. The degradation of cyclin B by the proteasome inhibits Cdk1 function, triggering mitotic progression into anaphase. Thus APC/C function has to be blocked until all the chromosomes are properly attached to the spindle. The APC/C is inhibited by the mitotic checkpoint complex (MCC). This group of proteins (Mad2, Mad3, and Bub3) binds to Cdc20, the activator of the APC/C, delaying anaphase until all the chromosomes are perfectly attached to the spindle. Other MC pathway proteins such as Mps1 kinase are required to recruit the components of the MCC to the kinetochores. Once chromosomes are properly attached, the inhibitory signal is no longer made, the APC/C is activated, and progression into anaphase begins.

The binucleate pathogen *Giardia intestinalis* is a diplomonad, a member of a highly divergent eukaryotic lineage that has an unusual actin cytoskeleton and cell cycle regulatory machinery (Paredez *et al.*, 2011; Gourguechon *et al.*, 2013). It is a widespread zoonotic parasite that causes more than 280 million symptomatic human infections worldwide per year. *Giardia* cysts differentiate (excyst) into motile trophozoites that proliferate, colonize, and attach extracellularly to the proximal small intestine, causing acute and chronic diarrheal disease by undefined mechanisms. Trophozoites then encyst in the distal part of the small intestine, and cysts are shed and ingested by new hosts. Regulation of mitosis and the cell cycle is required to proliferate and transition between the life cycle stages, yet we have a limited understanding of how these fundamental processes are regulated in this organism. *Giardia* has two diploid nuclei and undergoes mitosis in a manner similar to other eukaryotic cells, conserving a recognizable prophase, metaphase, anaphase, and telophase (Supplemental Figure S1). Despite this conservation, *Giardia* has a highly divergent spindle morphology. Each nucleus undergoes a semiopen mitosis in which each bipolar microtubule array surrounds the nucleus exterior (with an intact nuclear envelope), and spindle microtubules enter the nucleus through special pores to contact the kinetochores during prophase (Sagolla *et al.*, 2006). The two mitotic spindles assemble independently, but their functions are synchronized to separate chromosomes simultaneously. As in other eukaryotes, chromosomes are segregated both by forces generated at the kinetochore by microtubule depolymerization (Dawson *et al.*, 2007) and by forces that elongate the spindle on the nuclear envelope. Thus understanding the *Giardia* cell cycle is essential for the development of new drugs to treat giardiasis.

Cell cycle regulation in *Giardia* is not well described, and only recently have we begun to understand the molecular mechanisms controlling cell division in this divergent eukaryote. *Giardia* has conserved many of the components regulating the cell cycle in other organisms: cyclins, cyclin-dependent kinases (CDKs), Aurora and Polo kinases, PP1 and PP2 phosphatases, and separase. *Giardia* also has two components of the MCC, Mad2 and Bub3, and the regulatory kinase Mps1. However, other MC components are missing or so divergent in sequence that they are unrecognizable through bioinformatics studies. *Giardia* is missing most of the components required to make an inhibitory signal, including the pseudokinase BubR1/Mad3; the kinetochore protein Knl1, required to localize the MCC to the kinetochore in other eukaryotes; and the target of the MC pathway, the APC/C and its activator Cdc20 (unpublished data; Gourguechon *et al.*, 2013).

The absence of the APC/C and the reduced complexity of the remaining MCC components suggest that *Giardia* may not have a canonical MC and may lack a feedback loop that can regulate kinetochore function and mitotic progression. Here we show that morpholino knockdown of the expression of Bub3, Mad2, or Mps1 results in a lower mitotic index and chromosome missegregation. During interphase, the knockdown cells have just one nucleus or two nuclei with one of them misplaced. These results demonstrate that known MC components, even in the absence of the complete MC pathway, regulate spindle assembly and kinetochore function, and have a novel function: synchronization of mitosis between the two *Giardia* nuclei. Although Mps1 and Bub3 are associated with chromatin and centromeres during mitosis, Mad2 has a cytoplasmic location in association with spindle microtubules but not chromatin. This suggests that the homologues of the MC in *Giardia* regulate mitosis in two different ways: some proteins are associated with centromeres and required for kinetochore function, and others are associated with the cytoplasmic spindle microtubule array and are required for spindle assembly.

RESULTS

Giardial Mad2, Bub3, and Mps1 share sequence similarity with the MC proteins from other species

Sequence alignments of MCC proteins from *Homo sapiens*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae* were performed against the *Giardia* protein sequence database to find homologues of these proteins in this organism. Although there are at least eight major players in the MC pathway shared by humans and yeast (Mad1, Mad2, BubR1, Bub1, Bub3, Knl1, Mps1, and Cdc20), only Mad2, Bub3, and Mps1 could be identified in the *Giardia* genome (Table 1). It is possible that homologues of some of these components of the MC are present in *Giardia*, but with a sequence so divergent that this strategy is no longer suitable.

giMad2 (GL50803_100955) has the typical HORMA domain found in Mad2 proteins from other species. The HORMA domain (from Hop1p, Rev7p and Mad2) appears in proteins with functions related to the MC, chromosome synapsis, and DNA repair (Aravind and Koonin, 1998). The sequence identity between giMad2 and the protein in humans, *S. pombe*, and *S. cerevisiae* are 41, 33, and 38%, respectively, including conservation of three residues (R, E, and H) found in other proteins with HORMA domains and that appear to be invariant (Aravind and Koonin, 1998; arrows in Figure S2A).

The sequence identity of giBub3 (GL50803_102890) with the corresponding proteins in humans, *S. pombe*, and *S. cerevisiae* is 28, 24, and 22%, respectively. Domain analysis in giBub3 identifies WD40 domains. These WD40 domains are typically involved in protein–protein interactions and in the formation of multiprotein complexes (reviewed in Xu and Min, 2011). giBub3 conserves the WD residues typical of WD40 domains (underlined in red in Figure S2B).

We found an orthologous gene to the kinase Mps1, giMps1 (GL50803_4405), sharing 25, 30, and 24% identify with the C-terminal of *H. sapiens*, *S. pombe*, and *S. cerevisiae*, respectively. giMps1 conserves the amino acids required for the phosphate-binding site; the ATP-binding site; the hinge with the gatekeeper residue; the catalytic and activation loops, including the catalytic residues; and the autophosphorylation sites (Figure S3).

	H. sapiens	*S. pombe*	*S. cerevisiae*	*G. intestinalis*	*T. vaginalis*	*N. grubei*
Mad1	Mad1	Mad1	Mad1	—	Mad1	Mad1
Mad2	Mad2	Mad2	Mad2	Mad2	—	Mad2
BubR1	BubR1	Mad3	Mad3	—	—	Mad3
Bub1	Bub1	Bub1	Bub1	—	—	Bub1
Bub3	Bub3	Bub3	Bub3	Bub3	Bub3	Bub3
Knl1	Knl1	KNL1/Spc7	Spc105	—	—	—
Mps1	Mps1	Mph1	Mps1	Mps1 (TTK)	?	Mps1
Cdc20	Cdc20	Cdc20	Cdc20	—	—	Cdc20

TABLE 1: Comparison of MCC proteins in different organisms.

The knockdown of Bub3, Mad2, or Mps1 produces defects in chromosome segregation and nuclear number and positioning

To investigate the function of these three MC proteins, we used giardial cell lines containing a hemagglutinin (HA)-tagged version of each gene integrated into the genome under control of its own promoter and morpholino-mediated gene specific knockdown (Carpenter and Cande, 2009). We used PCR to confirm that the constructions with the HA tag were integrated into the genome in the correct position and under the endogenous promoter (Figure S4). After 48 h, the protein level of each protein was reduced by around 50% in cell populations as detected by Western blotting (Figure S5). Our previous experience with morpholino-mediated knockdowns has shown that not all the cells are going to take up the same amount of morpholino molecules; some cells will have little target protein, while others will have near-normal levels. All three lines grew more slowly than wild-type cells, with the largest reduction in growth rates observed in Bub3 knockdown cells, suggesting the MC genes are essential genes involved in the control of cell division (Figure 1A). Besides this problem, MC knockdowns show a lower mitotic index than wild-type cells. In our experiments, wild-type cells showed a mitotic index of 0.99% (n = 806 cells), Bub3-KD 0.17% (n = 1801 cells), Mad2-KD 0.28% (n = 1073 cells), and Mps1-KD 0.19% (n = 1042 cells).

Giardia intestinalis chromosome segregation occurs simultaneously on the two mitotic spindles, and sister chromatids are segregated to the left or right side of the cell at the same time (Figures S1 and 1B). In the Bub3, Mad2, and Mps1 knockdown cells, chromosome segregation is perturbed, with single chromosomes dispersed through the mitotic spindles in anaphase cells (Figure 1, C, E, and D, respectively). In the Mps1 knockdown cells we sometimes observe that chromosome segregation in one of the spindles is more similar to wild type than in the other, but in these cases we observe lagging chromosomes on both anaphase spindles (arrow showing lagging chromosome in Figure 1D). We also find defects in spindle morphology. The spindle poles are not as well defined as in wild-type cells, and the parallel orientation of the two spindles is lost. In the Mad2 knockdown, defects in spindle morphology are not as obvious, but appearance of the spindles differs from those of wild type (Figures 1E and 7E). In the Bub3 and Mps1 knockdown mitotic cells, it is very difficult to distinguish whether two separate spindles are present, even after careful examination of individual Z sections before making a projection (Figure 1, C and D). There are other examples, such as in the Bub3 knockdown, in which the mitotic spindle is not correctly formed and chromatin is not associated with the mitotic spindle (Figure 1C, bottom).

We also see defects in interphase cells that we interpret as occurring subsequent to the mitotic defects. For the knockdown of each gene, we observe cells with just one nucleus instead of two, and some cells with two nuclei, but with one mislocalized (Figure 2A for wild type and Figure 2B for the knockdowns). Although one can detect problems in nuclear number and position in wild-type cells, especially when the concentration of cells is very high and cells have reached confluency, quantification of these defects shows that the number of cells with abnormal nuclear distribution phenotypes in the knockdown strains is much higher, and the maximum number of cells with defects is seen 48 h after morpholino treatment (Figure 2, D and E). Again, the Bub3 knockdown has the strongest phenotype. In the examples of cells with two nuclei, with one of them misplaced, we observe a bias in distribution; most of the cells have the two nuclei on the left side of the cell (81% for Bub3 knockdown, 78% for Mad2 knockdown, and 81% for Mps1 knockdown). For the cells with just one nucleus, we see a similar bias, with most cells having the nucleus on the left side of the cell (91.6% for Bub3 knockdown, 89.6% for Mad2 knockdown, and 90.9% for Mps1 knockdown; Figure 2F).

In addition to the nuclear and spindle-associated defects, Mps1 knockdown cells show another defect: interphase cells that are larger than those in wild type. The two cell size classes are shown together in Figure 2: a normal-sized cell at the top and a larger cell at the bottom (arrow pointing to the bigger cell in Figure 2B, Mps1-KD row). For the Mps1 knockdown, there are more cells with an area greater than 120–140 μm^2 than in the wild-type control (Figure 3A). Interestingly, the "big cells" do not have defects in nuclear number and position. We are not able to find any big cell with nuclear defects, and as shown in Figure 3B, almost all cells with nuclear defects have an area <140 μm^2. This observation suggests that the two phenotypes (spindle-associated defects and increased size in interphase) are independent, and we speculate that they are generated by different levels of morpholinos in cells, resulting in different levels of Mps1 expression.

To determine whether defects in sister chromatid cohesion are responsible for most of the observed chromosome segregation errors following MC protein knockdown, we knocked down expression of two proteins in the cohesion complex, Smc1 and Smc3. These proteins were chosen following analysis of the *Giardia* genome searching for homologues of cohesion complex proteins (Figure S6). Smc1 and Smc3 were shown to be conserved in *Giardia*. We knocked down Smc1 and 3 with specific morpholinos and saw chromosome missegregation, but to a lesser degree than in the MC knockdowns. We did not see any defects or problems in mitotic spindle organization (Figure S7, A and B). This might indicate that

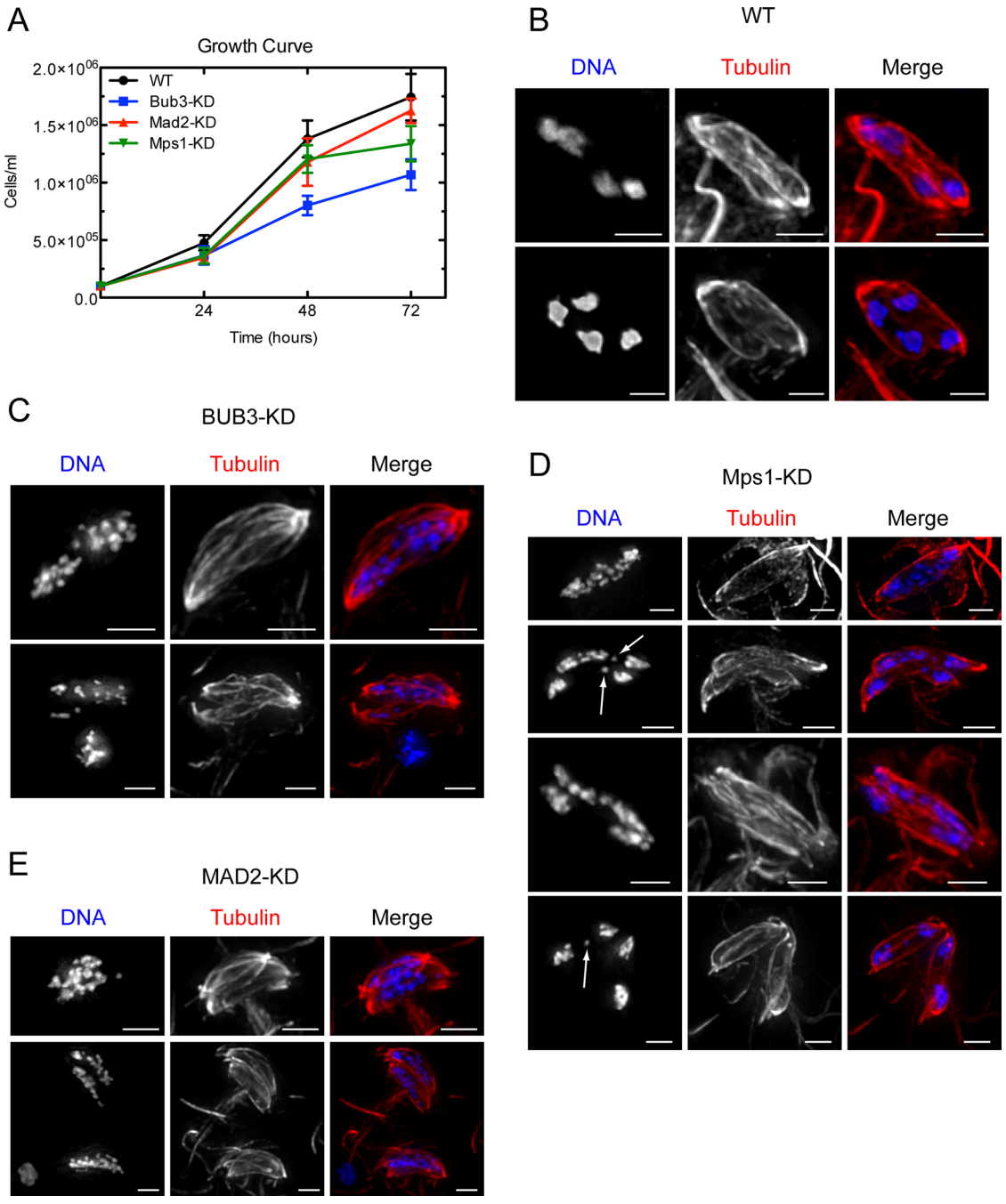

FIGURE 1: Cells after morpholino knockdown of either Mad2, Bub3, or Mps1 show defects in chromosome segregation, spindle morphology, and growth rate. (A) Growth curve of knockdown strains. *Giardia* cells were electroporated with morpholinos as described previously (Carpenter and Cande, 2009). Each electroporation was split in four tubes with defined media for replicates. At 24, 48, and 72 h, a sample was collected, and the number of cells was determined using a hemocytometer. Data are mean ± SD of three independent experiments. (B–E) *Giardia* mitotic spindles during anaphase. *Giardia* cells were fixed and immunostained with antibodies for tubulin and DAPI for DNA. Maximum-intensity Z-projections are presented. Scale bars: 2 μm. (B) wild type (WT), (C) Bub3 knockdown, (D) Mps1 knockdown, and (E) Mad2 knockdown.

the defects in the MC knockdowns affect functions that are upstream of cohesin complex function.

MC protein localization

In metazoan and yeast cells, MC proteins localize at the kinetochores during early phases of mitosis to inactivate the APC/C and delay cell cycle progression until all the chromosomes are properly attached to spindle microtubules. They are then released from the kinetochore during the transition from metaphase to anaphase or after proper bipolar chromosome orientation is established (Kops and Shah, 2012). We used the HA-tagged version of the *Giardia* proteins Mps1, Bub3, and Mad2 to determine whether they behaved similarly to their human and yeast counterparts during the cell cycle.

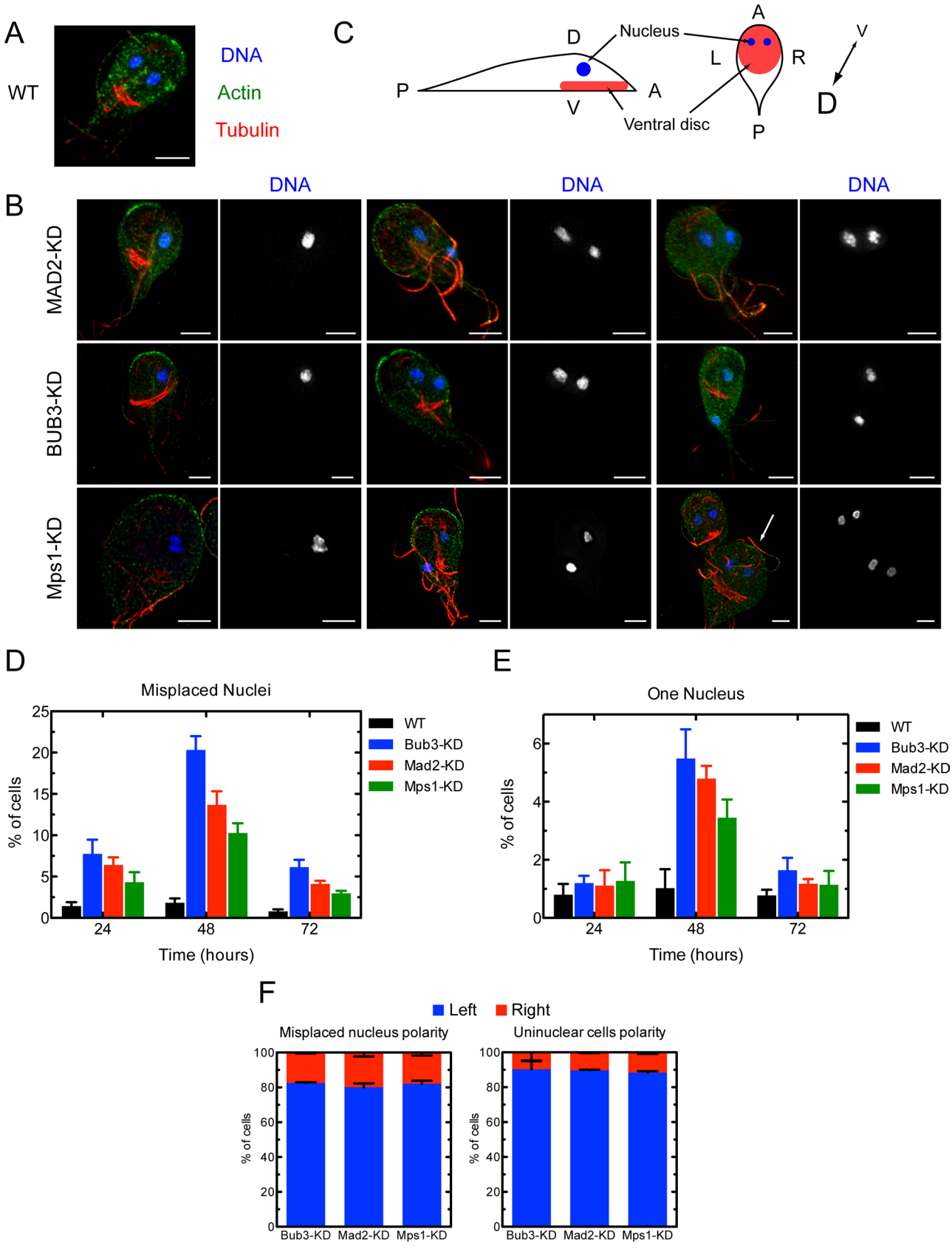

FIGURE 2: Interphase cells of Mad2, Bub3, or Mps1 knockdowns show defects in nuclei number and positioning. (A and B) *Giardia* cells were treated with morpholinos, collected after 48 h, and fixed for immunofluorescence as described in *Materials and Methods*. DNA was stained with DAPI (blue), actin (green), and tubulin (red). We show just the DAPI channel next to the merge images to show the number of nuclei. All the images are maximum-intensity Z-projections. Scale bars: 4 μm. The arrow in B (Mps1-KD row) points to a cell with bigger size than wild type. (C) Diagram showing a

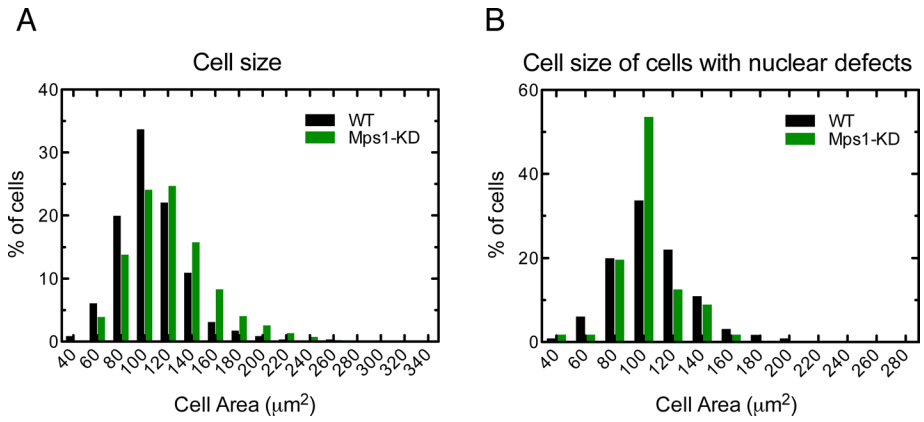

FIGURE 3: Mps1 knockdown cells present a large cell size defect. (A) Cell size of wild-type and Mps1 knockdown cells. Cells were treated with morpholinos, collected after 48 h, and fixed for immunofluorescence with antibodies against actin. Pictures of random fields of the slides were taken with a Deltavision system. Cell size was determined using ImageJ software. Histograms were done using Prism software. $n = 575$ cells for wild type and $n = 817$ cells for Mps1-KD. (B) Cell size of Mps1 knockdown cells with nuclei defects. Cells were treated with morpholinos, collected after 48 h, and fixed for immunofluorescence with antibodies against actin. Pictures of nuclear defective cells in the Mps1 knockdown sample were taken with a Deltavision system. Cell size was determined using ImageJ software. Histograms were done using Prism software. $n = 575$ cells for wild type and $n = 56$ cells for Mps1 knockdown.

Bub3

The Bub3-HA protein is located inside the nucleus when the chromatin begins to condense to form discrete chromosomes. Once the chromosomes are condensed, Bub3-HA localizes as a small number of dots on chromosomes (Figure 4, B and C), a localization that resembles a centromeric localization. Once the cells advance further into mitosis, the chromatin localization disappears (unpublished data). To check the specificity of this localization, we compared these results with a non-tagged wild-type strain (Figure 4A) and with Bub3-HA cells treated with morpholinos against Bub3. Bub3-HA signal is undetectable in cells with spindle or interphase defects after morpholino treatment (Figure S8, A and B).

Mad2

Mad2-HA protein is on the median body in interphase cells (Figure 4D). During interphase and when the chromatin begins to condense, some Mad2-HA signal appears on the two internal axonemes between the two nuclei (arrows in Figure 4D). At the beginning of mitosis, there is a weak, but clear association of Mad2 with the cytoplasmic spindle microtubules, but none is inside the nucleus (arrow in Figure 4E). This localization to the spindle disappears as the cells advance into anaphase (Figure 4F). During late stages of mitosis, Mad2-HA signal has almost completely disappeared from the cell (Figure 4G). As is the case for Bub3, we do not see Mad2-HA in cells treated with Mad2 morpholinos that display a phenotype (Figure S8, C and D).

Mps1

Mps1-HA is found as a rim around the nuclear periphery during interphase (Figure 5, interphase cell). This localization resembles that of proteins in the nuclear pore (Figure S7C). As *Giardia* cells progress into mitosis, the Mps1 localization changes dramatically. As chromatin begins to condense to form discrete chromosomes, the peripheral nuclear signal begins to decrease, and relocalizes to the chromosomes (Figure 5, condensed chromosomes). During prophase and metaphase, the perinuclear staining disappears, and Mps1 is now localized as a small number of discrete foci on chromosomes, resembling kinetochore localization. During anaphase, Mps1-HA moves to the cytoplasmic spindle microtubules, and during telophase, Mps1-HA accumulates in the center of the two spindles. During cytokinesis, the perinuclear distribution of Mps1 is reestablished. This behavior is identical for that observed for Aurora kinase (Gourguechon and Cande, personal communication). No Mps1 signal is observed in the cells treated with Mps1 morpholinos that display a phenotype (Figure S8, E and F, for cells with nuclear defects, and G, for Mps1 knockdown big cell).

In metazoans and yeast, Mps1 is upstream of Mad2 and Bub3 during MC activation. Mps1 is required for Mad2 localization in these cells, probably helping to establish the proper localization of Mad1 (Martin-Lluesma *et al.*, 2002; Liu *et al.*, 2003). Mps1 may also play a direct role in localization of Bub3 (Yamagishi *et al.*, 2012). To understand the hierarchy of interactions of MC proteins in *Giardia*, we analyzed the localization of each protein in cells treated with morpholinos against the other proteins. Cells treated with morpholinos for Mad2 or Bub3 do not have defects in Mps1 localization. Mps1 localization remains unperturbed in Mad2 and Bub3 knockdown cells despite nuclear number and position defects (Figure S9). However, Mad2 and Bub3 localization is lost in cells treated with Mps1 morpholinos. We do not see Bub3 localization in the nucleus (Figure 6, A and B) or Mad2 localization in the median body during interphase (Figure 6, C and D).

Giardia cell from a lateral and a dorsal view to define right and left sides of the cell (A, anterior; P, posterior; D, dorsal; V, ventral; R, right; L, left). (D and E) Percentage of cells with two nuclei, but one of them misplaced from its proper position (D), and number of cells with just one nucleus (E) after the morpholino treatment for the hours indicated. Cells were collected, fixed for immunofluorescence, and stained as previously described. Data are mean ± SD for three independent experiments. Wild type (WT; $n = 1255$ cells at 24 h, $n = 1161$ at 48 h, and $n = 1151$ at 72 h). Bub3-KD ($n = 1077$ at 24 h, $n = 1268$ at 48 h, and $n = 1082$ at 72 h). Mad2-KD ($n = 1027$ at 24 h, $n = 1203$ at 48 h, and $n = 1012$ at 72 h). Mps1-KD ($n = 1170$ at 24 h, $n = 1358$ at 48 h, and $n = 1218$ at 72 h). (F) Quantification of cell polarity. *Giardia* cells were treated with morpholinos during 48 h, collected, and fixed for immunofluorescence as previously described. To determine the right and left position of the nucleus, we examined the *z*-axis of *Giardia* cells to find the dorso-ventral position of the cell. Once we knew whether the ventral disk was closer to the slide surface or closer to the coverslip, we defined the left and right side as it was defined previously in our lab (Sagolla *et al.*, 2006) and according to C. Data are mean ± SD for three independent experiments. Misplaced nucleus cells: $n = 101$ cells for Bub3 knockdown, $n = 101$ for Mad2 knockdown, and $n = 104$ for Mps1 knockdown. Uninuclear cells: $n = 24$ for Bub3 knockdown, $n = 29$ for Mad2 knockdown, and $n = 22$ for Mps1 knockdown.

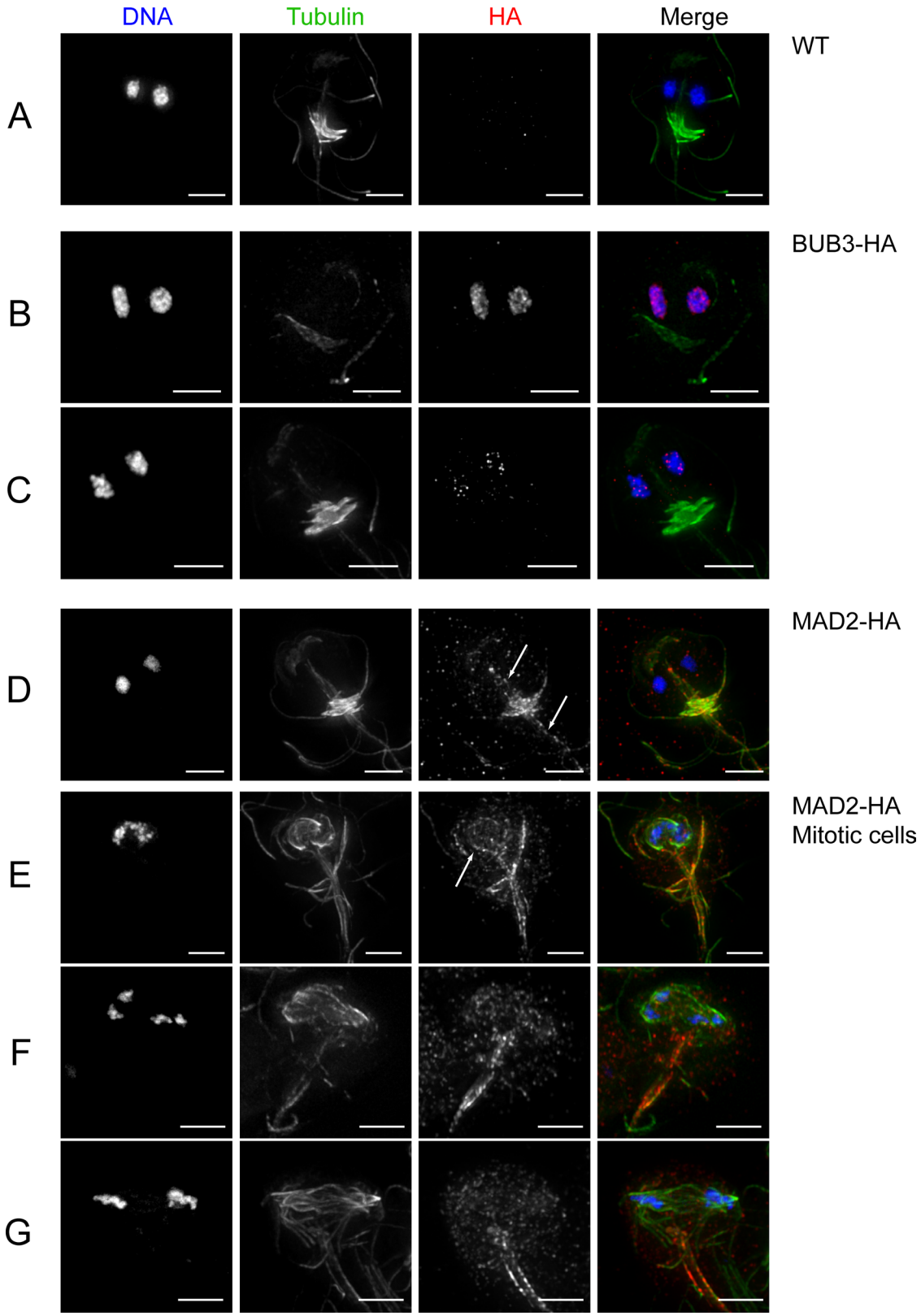

FIGURE 4: Bub3-HA and Mad2-HA localization during *Giardia* cell cycle. DNA was stained with DAPI (blue), tubulin-zenon antibody (green), and HA antibody (red). All the images are maximum-intensity Z-projections. Scale bars: 4 μm. (A) Wild-type cells as control. (B and C) Bub3-HA localizes inside the nucleus in dots in condensed chromosomes. (D) Mad2-HA localizes to the medium body and axonemes between the nuclei (arrows point to the internal axonemes). (E–G) Mad2-HA in mitotic cells. At the beginning of mitosis (E), we can detect some signal associated with the spindle microtubules (arrow in E). During anaphase (F) and telophase (G), the signal from the spindle disappears and we can just detect signal in the central axonemes.

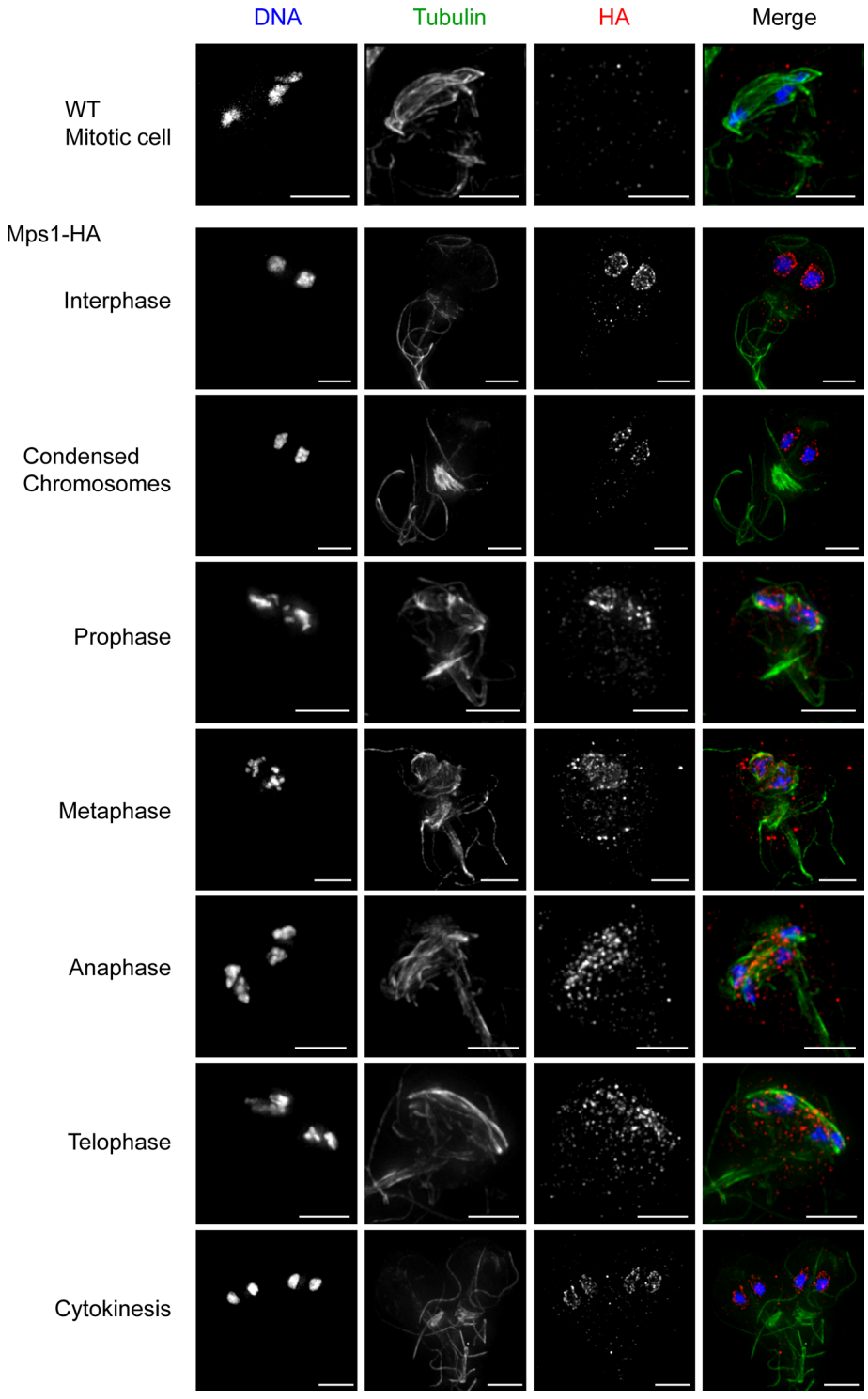

FIGURE 5: Mps1-HA localization during *Giardia* cell cycle. DNA was stained with DAPI (blue), tubulin-zenon antibody (green), and HA antibody (red). All the images are maximum-intensity Z-projections. Scale bars: 4 μm. Mps1-HA localizes in a rim around the nucleus during interphase. When the cells enter mitosis, the localization around the nucleus disappears, and we can see signal inside the nucleus on condensed chromosomes. During anaphase and telophase, Mps1-HA signal seems to be in the mitotic spindle and then to return to the nuclear envelope during cytokinesis.

Spindle pole organization

To check whether the defects seen in the knockdown cells are related to problems in spindle formation/behavior, we monitored spindle pole behavior in MC morpholino-treated cells. Centrin is a calcium-binding protein that binds to microtubules in the centrosomes of the microtubule-organizing center (MTOC) in most eukaryotic cells. Centrin is known to be important for centriole duplication, MT dynamics, and spindle formation (Salisbury, 1995, 2007; Schiebel and Bornens, 1995; Rice and Agard, 2002; Dantas *et al.*, 2012). Centrin is phosphorylated by Mps1, and lack of Mps1 results in problems in centrosome duplication and malformation of the mitotic spindle in many cell types, often seen as monopolar and multipolar spindles (Araki *et al.*, 2010; Yang *et al.*, 2010). We wanted to determine whether the defects in spindle morphology seen in the MC protein knockdowns were associated with a problem in centrin behavior. *Giardia* centrin has been shown to localize to basal bodies (Belhadri, 1995; Meng *et al.*, 1996; Corrêa *et al.*, 2004; Sagolla *et al.*, 2006). *Giardia* has four pairs of basal bodies located between the two nuclei of interphase cells, and the four pairs of internal flagellar axonemes (anterior, posterior, ventral, and caudal) are nucleated by these basal bodies (Nohýnková *et al.*, 2000, 2006; Maia-Brigagão *et al.*, 2013). During mitosis, the basal bodies and their associated axonemes migrate to the periphery of the two nuclei and are incorporated into the spindle poles (Sagolla *et al.*, 2006; Tůmová *et al.*, 2007). In interphase wild-type cells, there are two groups of four dots between the nuclei, corresponding to the eight basal bodies (Figure 7A; Belhadri, 1995; Meng *et al.*, 1996; Corrêa *et al.*, 2004; Sagolla *et al.*, 2006). In defective interphase cells in Mps1 and Mad2 knockdown strains, we observed some cells with half the normal centrin signal (Figure 7D for Mad2 and Figure 7F for Mps1). This centrin loss does not occur at random, and these cells have lost the signal for the four basal bodies that are normally on the same side of the cell (Figure 7D). These cells have only four flagella instead of the regular eight. This observation suggests that when cells lose centrin from one side, they then lose one of each of the flagellar pairs, resulting in cells with just one anterolateral, one posterolateral, one ventral, and one caudal flagellum. Centrin loss does not occur in all the cells, and some nuclear defective cells show normal centrin levels (unpublished data). In wild-type anaphase cells, centrin stains the two poles of the mitotic spindle: there are two distinct dots on both sides of a spindle (Figure 7B). Bub3 knockdown cells do not show a clear defect in the position or number of centrin spots in the spindle poles, but do show some

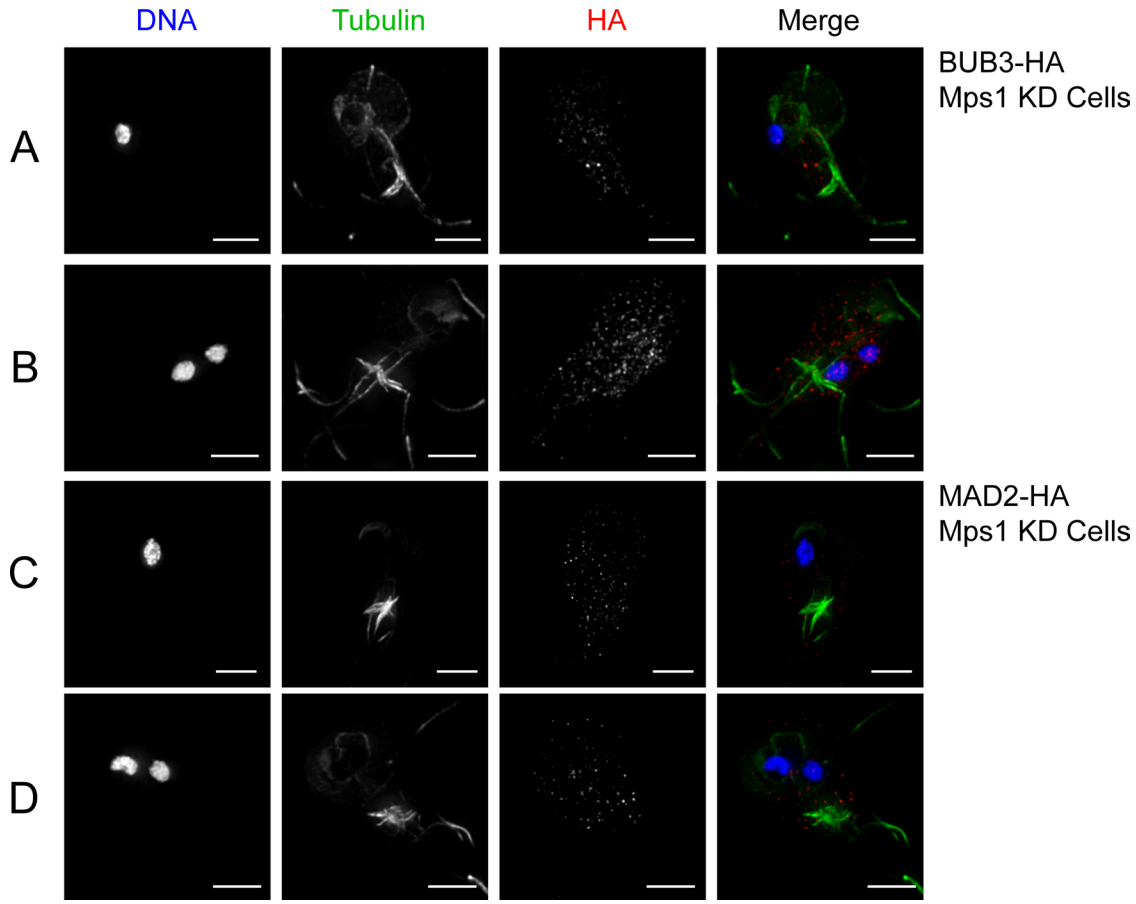

FIGURE 6: Mad2-HA and Bub3-HA localization is lost in Mps1 knockdown cells. DNA was stained with DAPI (blue), tubulin-zenon antibody (green), and HA antibody (red). All the images are maximum-intensity Z-projections. Scale bars: 4 μm. (A and B) Bub3-HA signal in Mps1-KD interphase cells. We do not see any Bub3-HA signal inside the nucleus in defective cells. (C and D) Mad2-HA signal in Mps1 knockdown interphase cells. The Mad2-HA localization is lost from the medium body in cells with nuclear defects.

elongated structures coming out of the spindle poles (Figure 7C). In the morpholino knockdowns for Mad2 and Mps1, we find defects in centrin localization and number of dots associated with the spindle poles during mitosis (Figure 7E for Mad2 and Figure 7, G and H, for Mps1). Centrin misdistribution may be responsible for the defects in spindle morphology and spindle orientation in these cells.

DISCUSSION

MC in *Giardia*

Eukaryotic cells have evolved multiple mechanisms to ensure proper chromosome segregation during mitosis. The SAC/MC, whose components are associated with the chromatin at or near the kinetochore, halts progression into anaphase until all chromosomes have achieved a bipolar attachment to the microtubules emanating from each pole of the mitotic spindle. Without this control system, cells are unable to sense merotelic kinetochore attachment to the spindle and can initiate anaphase-generating chromosome missegregation leading to aneuploidy.

The molecular mechanism responsible for the MC is highly conserved among eukaryotes and involves regulation of the E3 ubiquitin ligase complex APC/C, whose activation is required to enter anaphase. The main components involved in the inactivation of the APC/C are Bub3, Mad2, and Mad3, known as the MCC. These components are recruited to the kinetochore during mitosis, and they form a signal to inactivate the APC/C by binding to the APC/C activator Cdc20. The MCC protects cyclin B and securin from ubiquitination by the APC/C and subsequent degradation by the proteasome, hence blocking cell cycle progression and release of sister chromatid cohesion by separase. Besides these three players, other proteins in the kinetochore function to activate or recruit MCC components at the kinetochore. These include Aurora B and Mps1 kinases, Mad1, and the kinetochore structural protein Knl1. Although the MCC proteins are very well conserved among eukaryotic cells, *Giardia* lacks many components of this pathway, including Mad1; the pseudokinase BubR1 (Mad3 in yeast), which is involved in activating the inhibitory signal; and Knl1, which anchors the MCC to the kinetochore.

With many of the canonical MC components missing, does *Giardia* have an actual MC? To address this question, we need to understand the consequences of the absence of MC components in organisms with a canonical MC. The first reports describing the components of the MCC are from the 1990s, when several groups discovered the MCC and the Mps1 kinase in genetic screens while studying cells that were able to bypass the metaphase/anaphase transition checkpoint (Hoyt *et al.*, 1991; Li and Murray, 1991; Weiss and Winey, 1996). In later studies, several groups showed that low levels of Mad2 or Bub3 generate chromosome missegregation (Dobles *et al.*, 2000; Kalitsis *et al.*, 2000; Shonn *et al.*, 2000; Michel *et al.*, 2001; Warren *et al.*, 2002; Babu *et al.*, 2003; Cheslock *et al.*, 2005; Homer *et al.*, 2005; Lopes *et al.*, 2005) and a lower mitotic index (Dobles *et al.*, 2000; Kalitsis *et al.*, 2000; Michel *et al.*, 2001),

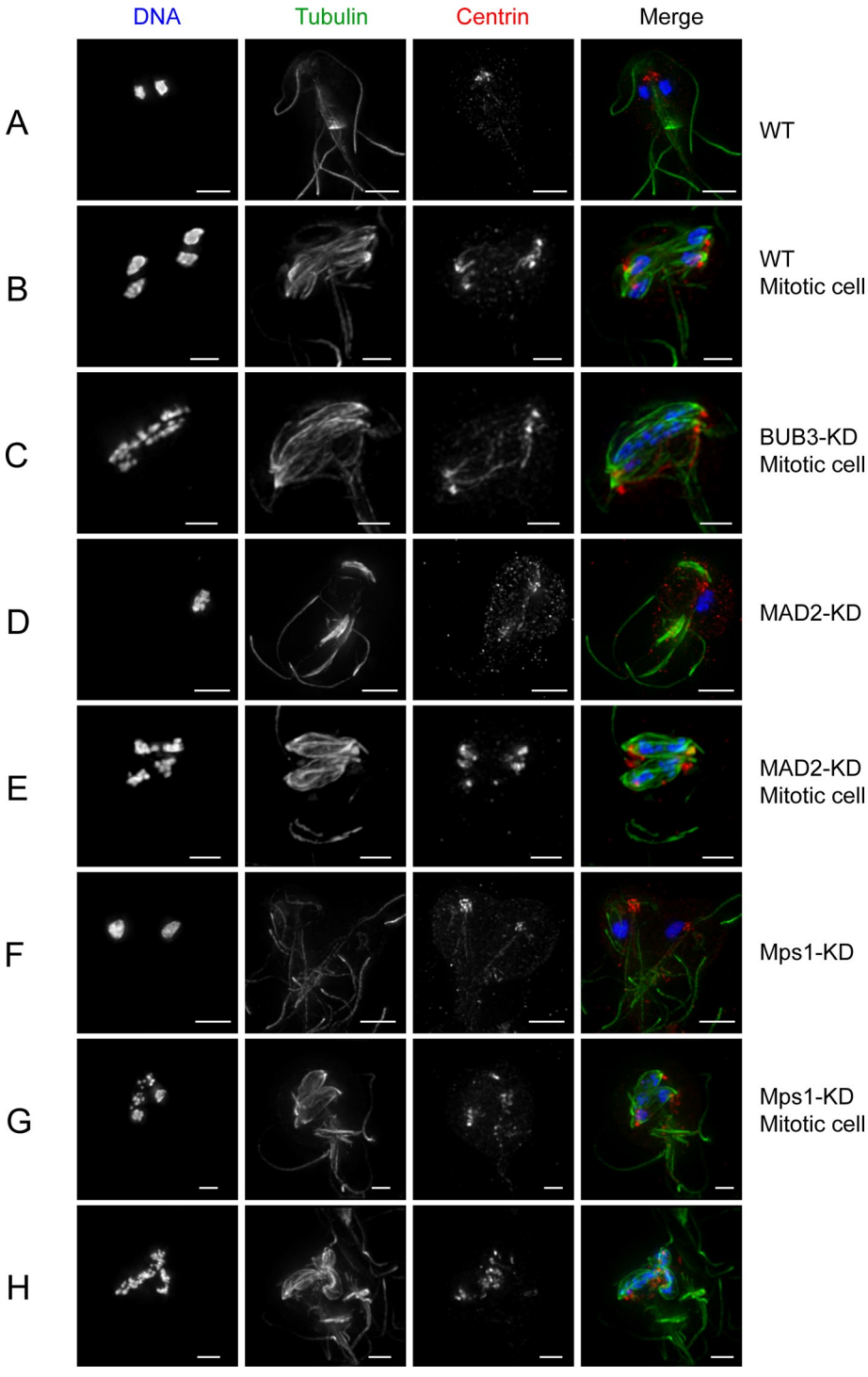

FIGURE 7: Centrin is mislocalized in the MC knockdowns. DNA was stained with DAPI (blue), tubulin-zenon antibody (green), and centrin antibody (red). All the images are maximum-intensity Z-projections. (A) Centrin localization in interphase wild-type cells. The centrin signal is located between the two nuclei. Scale bars: 4 μm. (B) Centrin localization in a mitotic wild-type cell. We can see the four foci of centrin making the spindle poles. Scale bars: 2 μm. (C) Bub3 knockdown cells show four foci of centrin at the spindle poles, but they also show long filaments coming from the spindle poles and connecting with the central axonemes. Scale bars: 2 μm. (D) Centrin localization in interphase Mad2 knockdown cells. In these cells, half of the centrin staining and half of the flagella have been lost. Scale bars: 4 μm. (E) Mad2 knockdown mitotic cells show aberrant centrin foci number and location. Scale bars: 2 μm. (F) Centrin localization in interphase Mps1 knockdown cells. The cell in the right has lost half of its centrin. It is not clear whether the cell on the left has normal centrin levels. Scale bars: 4 μm. (G and H) Mps1 knockdown mitotic cells show defects in centrin foci localization and number. Scale bars: 2 μm.

and that loss of Bub1 or Bub3 increases the rate of chromosome segregation errors (Pangilinan and Spencer, 1996; Warren *et al.*, 2002; Lampson and Kapoor, 2005).

In *Giardia*, the knockdown of Bub3, Mad2, or Mps1 reduces the mitotic index and generates cells with chromosome missegregation; phenotypes that match the phenotypes in other organisms with an impaired MC. Although the morpholino phenotypes are consistent with the presence of an MC, *Giardia* lacks an APC/C, that is, the target for the inhibitory signal, thus it is unlikely that *Giardia* has a feedback loop for error correction. This suggests that *Giardia* has a noncanonical MC unable to arrest the cells in the metaphase to anaphase transition to allow error correction. In support of this argument, we observe that *Giardia* has low levels of chromosome missegregation in wild-type populations that are tolerated because it is a tetraploid organism and may have infrequent meiosis (Tůmová *et al.*, 2007). In mammalian cells, microtubule poisons like vinblastine and Taxol are able to activate the MC, inducing an arrest in metaphase (Gorbsky, 2001). *Giardia* cells treated with Taxol showed no increase in the number of mitotic cells or cells arrested in metaphase (unpublished data). The use of other drugs such as nocodazole and aphidicolin neither increased the mitotic index nor arrested the cells in mitosis; rather, cells arrest in G2 or G1/S phase (Poxleitner *et al.*, 2008). With this in mind, we propose that *Giardia* lacks a classical MC, as it is missing the components required to generate the inhibitory signal that blocks cell cycle progression in metaphase, and it may not be able to sense proper attachment of the kinetochores to the microtubules. Instead, we propose that the MC proteins act to trigger and regulate spindle formation and behavior but cannot halt mitosis. We propose that the MC components conserved in *Giardia* are involved directly in the regulation of kinetochore function and spindle assembly potentially by activating the function of spindle kinesins and kinetochore proteins such as Nuf2 and Kin13, which interact with spindle microtubules. But it is not a classical MC such as that described for eukaryotic cells like human and yeast cells, as it lacks the components required for feedback inhibition of spindle function.

Giardia has a minimalist MC pathway; this pathway is either reduced by loss of components from the more complex pathways found in yeast and humans or it may represent an ancient simplified mechanism to control chromosome segregation. It regulates spindle functions required for chromosome

segregation but cannot sense or correct the errors in spindle function that may lead to chromosome missegregation. It is possible that the canonical MC found in most eukaryotes has evolved from this simplified pathway. There are two sister groups to *Giardia* in the Excavate kingdom that also have a reduced MC: *Trichomonas vaginalis*, a parabasalid, and *Naegleria grubei*, a heterolobosean. *T. vaginalis* has Bub3, Mad1, and a kinase similar to Mps1 in the genome, but no APC/C (Table 1). However, the heterolobosean soil amoeba, *N. grubei*, which is in a more distantly related excavate group than is *T. vaginalis*, has most of the MC proteins, including Mad1, Mad2, Bub1, Bub3, Mps1, Cdc20, and some components of the APC/C (Table 1). Regardless of whether the APC/C was lost during the evolution of *Giardia* or was never present, it is clear from our data that *Giardia* has a chromosome segregation control system for which an APC/C is not required.

How are Bub3 and Mad2 localized in *Giardia* without Mad1 and Knl1?

Mad2. Mad2 does not behave like a typical checkpoint protein in *Giardia* in that it never associates with the kinetochores. In our experiments, Mad2 localizes to the internal caudal axonemes between the nuclei, on microtubule structures close to the basal bodies during interphase, and with the cytoplasmic spindle microtubule arrays during mitosis. It is possible that Mad2 migrated to its perinuclear position during prophase as the basal bodies and their associated axonemes migrate to the nuclear envelope to form the spindle poles. In another excavate, *Trypanosoma brucei*, a Mad2–yellow fluorescent protein construct does not localize inside the nucleus to kinetochores (Akiyoshi and Gull, 2013). In this organism, Mad2 localizes to the basal bodies, distant from the nucleus but close to the kinetoplast (mitochondrial DNA-containing organelle) at all phases of the cell cycle (Akiyoshi and Gull, 2013). It is possible that the function of Mad2 in trypanosomes is to control and monitor the segregation of the basal bodies and control kinetoplast division. Our observations suggest that giMad2 like the trypanosome Mad2 is an atypical MC protein. In *Giardia*, it regulates chromosome segregation and mitosis by controlling basal body function and the formation of the spindle. Moreover, the localization of giMad2 during the cell cycle resembles the localization of two spindle-associated kinesins, kinesin-13 (Dawson *et al.*, 2007) and kinesin-5 (unpublished data from Cande and Dawson labs). During interphase, kinesin 13, a regulator of microtubule plus-end dynamics, localizes to the median body and the caudal internal axonemes between the two nuclei, and during mitosis, it localizes to the mitotic spindle and kinetochores. If Mad2 regulates kinesin-13 activity, low levels of Mad2 could affect chromosome segregation and spindle formation. Kinesin-5 is also localized to the caudal axonemes between the two nuclei during interphase and at the poles of the mitotic spindle during mitosis (unpublished data from Cande and Dawson labs). It is possible that Mad2 may regulate the function of these two kinesins and thereby control the behavior of the spindle during mitosis. As in other organisms, Mps1 is required for Mad2 localization in *Giardia*. We were unable to detect a normal Mad2 signal in an Mps1 knockdown. We are unsure of how Mps1 is able to affect Mad2 localization, as they are not in the same cellular compartments, but it is clear that the Mad2-HA signal disappears from the median body in the Mps1 knockdown cells.

Bub3. In contrast to GiMad2, GiBub3 behaves like MC proteins in yeast and humans, it localizes to the kinetochores when chromosomes condense during prophase and is lost from the kinetochores during mitotic progression into anaphase. But how is Bub3 located to the kinetochore without Knl1? We have not been able to identify homologues to Knl1, either because there is no Knl1 or because the sequence is too divergent to identify the protein through bioinformatics. It is possible that Bub3 is anchored to the kinetochore without requiring Knl1 homologues. In other organisms, Mps1 is important for the localization of Bub3 and the pseudokinase BubR1 (Yamagishi *et al.*, 2012). In *Giardia*, Mps1 is required for Bub3 localization to the kinetochore, as we observe in the Mps1 knockdown that Bub3 is lost from the nucleus.

The different localization of these two proteins would imply that *Giardia* has two different sets of MC proteins: Mad2 is a cytoplasmic protein controlling mitosis through the regulation of the spindle assembly behavior, whereas Bub3 regulates kinetochore function in the nucleus. The different and milder mitotic phenotypes of the Smc1/Smc3 knockdown cells suggest that neither class of MC proteins directly regulates SCC. This is consistent with the unconventional morphology of the *Giardia* spindle. The spindle poles and polar microtubule arrays are in the cytoplasm, whereas the kinetochores and the kinetochore-attached microtubules are inside the nucleus (Sagolla *et al.*, 2006).

MC morpholinos affect cell polarity and nuclear number and position

What is the link between nuclear migration/positioning and the MC pathway? Previous FISH (fluorescence in situ hybridization) data from our lab (Sagolla *et al.*, 2006) support a nuclear migration model whereby cytokinesis occurs in the anterior–posterior plane of the cell, and nuclei are inherited with mirror image symmetry in each daughter cell.

Centrin is a Ca^{2+}-binding protein associated with centrioles and basal bodies of eukaryotic cells, and its activity is regulated in part by Mps1 in many cell types (Schiebel and Bornens, 1995; Rice and Agard, 2002; Beisson and Wright, 2003; Salisbury, 2007; Yang *et al.*, 2010; Dantas *et al.*, 2012). Centrin was discovered in flagellate green algae as part of the flagellar rootlet, an MTOC structure associated with the algal basal bodies (Salisbury *et al.*, 1984; McFadden *et al.*, 1987). In mammalian cells, the centrosomes (containing two centrioles at right angles to each other) act as an MTOC and help to organize and position the mitotic spindle (Bornens, 2012). In mammalian cells, centrin is required for centriole duplication (Salisbury *et al.*, 2002). In other eukaryotic cells that lack a traditional MTOC, such as *Chlamydomonas*, the organization and positioning of the mitotic spindle is defined by the flagellar basal bodies. The association of the basal bodies with the spindle poles assures that each daughter cell inherits half of the basal bodies required to form the flagellum. In *Giardia* eight basal bodies are located between the two nuclei. In previous work, it has been shown that these basal bodies migrate and are incorporated into the spindle poles (Nohýnková *et al.*, 2000; Sagolla *et al.*, 2006). We suggest that spindle formation and orientation, and nuclear positioning in the two daughter cells is determined by the behavior of the basal bodies and their associated structures. The position of these basal bodies close to the spindle poles can serve to orientate spindle position. Thus errors in basal body position would generate cells with problems in spindle orientation and eventually yield cells with abnormally placed nuclei. This is supported by the observation that Mps1 morpholino knockdown cells with one nucleus have half the normal number of flagellum and basal bodies when compared with cells with two nuclei. According to the flagellar maturation hypothesis (Nohýnková *et al.*, 2006), the most mature flagella reside on the left side of the cell. If the retained nucleus in mononucleated cells is associated with the mature flagella, this would explain the left-side bias in retained nuclear position that we observe.

How can we relate these observations to Mps1, Mad2, and Bub3 function? We know that Mps1 is required for Bub3 and Mad2 localization, suggesting that Mps1 functionally is upstream of these two proteins. Mps1 phosphorylates centrin in mammalian cells and yeast (Araki *et al.*, 2010; Yang *et al.*, 2010). In yeast, Mps1 is important for the interaction of centrin and Kar1, a protein involved in karyogamy and spindle pole body (SPB) duplication (Araki *et al.*, 2010). Mps1 mutants in yeast have problems in SPB duplication, generating monopolar spindles. In *Dictyostelium discoideum*, centrin (CenB) localizes to the nucleus and the knockout generates multinuclear cells, and it is proposed that this protein is involved in the maintenance of the nuclear architecture (Mana-Capelli *et al.*, 2009). In *Leishmania*, the knockout of centrin generates multinucleate cells with problems in basal body duplication (Selvapandiyan *et al.*, 2004). In *T. brucei*, centrin is involved in karyokinesis, and in mutant strains, there are two different populations of daughter cells: one without a nucleus and the other multinucleated (Shi *et al.*, 2008). Thus centrin in *T. brucei* is important for nuclear inheritance. In chrysomonads, centrin is one of the main components of the rhizoplast, a fibrous structure that physically connects the nucleus and the basal bodies. During mitosis in these protozoans, this fibrous structure connects the spindle poles to the basal bodies (Brugerolle and Mignot, 2003). *Chlamydomonas* is another example wherein centrin links basal bodies to the nucleus (Geimer and Melkonian, 2005).

Thus it is possible that nuclear migration/position and basal body migration/position are linked by centrin function, and any alteration in centrin behavior would affect the behavior of the basal bodies and the nucleus during mitosis. giCentrin conserves the residues required for Mps1 phosphorylation, so it is possible that Mps1 is controlling spindle formation and interphase nuclear positioning in *Giardia* via phosphorylation of centrin. Mps1 is perinuclear during interphase, a localization that is similar to that of the nuclear pore complex (Figure S7C). There are other examples in which Mps1 localizes to the nuclear envelope. In the closed mitosis of *S. cerevisiae*, Mps1 localizes to the SPB, a structure embedded in the nuclear envelope that organizes the mitotic spindle and affects nuclear positioning and chromosome segregation (Weiss and Winey, 1996; Castillo *et al.*, 2002; Jaspersen and Winey, 2004). In human cells, Mps1 localizes to the nuclear pore during interphase (Liu *et al.*, 2003). We cannot determine whether giMps1 is inside or outside the nucleus during interphase, but it is possible that Mps1 can phosphorylate a pool of perinuclear centrin that is then associated with the basal bodies at the beginning of mitosis to set up the mitotic spindle. In vertebrate cells, centrin is phosphorylated early in mitosis, when the new centrosomes are going to form the spindle, and the levels of phosphorylated centrin (P-centrin) are kept constant until metaphase, and then diminish to basal levels (Lutz *et al.*, 2001; Salisbury, 2007; Yang *et al.*, 2010). Thus P-centrin would be required to set up the mitotic spindle, but its levels would drop once the mitotic spindle is formed and ready to operate. We propose that the defect seen in chromosome segregation in the knockdown for Mad2 and Mps1 are due to problems in mitotic spindle setup/behavior linked to centrin, while the defects in chromosome segregation seen in Bub3 are due to problems in kinetochore assembly/behavior. This is supported by the fact that some Mad2 and Mps1 knockdown cells lack half the normal distribution of centrin, while this defect is not seen in Bub3 knockdown cells.

The multiple phenotypes of Mps1 knockdown cells suggest this protein has multiple functions during the cell cycle. As in other organisms, Mps1 could function at the G2/M transition. It is possible that Mps1 is important to start mitosis, and the lack of Mps1 activity halts the cells in the G2/M transition. This would explain the large-cells phenotype in the Mps1 knockdown. The second function would be to control spindle formation and chromosome segregation. Mps1 may phosphorylate centrin, and the lack of Mps1 could lead to misbehavior of centrin causing errors in nuclei number and/or position. Later in mitosis, Mps1 may affect chromosome segregation through Mad2 and Bub3.

We conclude that the MC proteins in *Giardia* play an important role in regulating spindle formation, chromosome formation, and indirectly, cell polarity, even in the absence of an APC/C and a feedback loop that blocks mitotic progression in the absence of proper chromosome attachment. We suggest that these represent the evolutionary conserved functions of the MC network and that the error-detection feedback loop may be a later evolutionary invention. Although it is possible that the APC/C was lost since diplomonads diverged from other eukaryotic lineages, our hypothesis is supported by the absence of an APC/C in several other Excavate groups in addition to *Giardia*.

MATERIALS AND METHODS

Strain, culture conditions, and morpholino knockdowns

G. intestinalis, strain WBC6 was cultured as described by Sagolla *et al.* (2006). Knockdown experiments were performed as described by Carpenter and Cande (2009) with specific morpholino oligos designed by Gene Tools (www.gene-tools.com). To design the morpholinos, we submitted the sequence of each gene to Gene Tools (Philomath, OR).

Construction of HA strains

HA-tagged constructions for each gene were made according to Gourguechon and Cande (2011), with the HA epitope tag in the C-terminal of each protein. A C-terminal region of each protein was amplified by PCR with specific oligonucleotides. For Bub3, we used the last 414 nt of the gene, the last 432 for Mad2, and the last 473 for Mps1. The PCR fragments were cloned in-frame in the pKS-3HA vector previously described by Gourguechon and Cande (2011). The construction with the c-terminal of each gene fused to the HA tag was linearized and electroporated in *Giardia* cells. Cell resistant to neomycin were selected and genomic DNA was extracted and tested by PCR for integration of the construction in the genome.

Fixation, immunofluorescence, and fluorescence microscopy

Fixation was done as described by Paredez *et al.* (2011). Cells were centrifuged 5 min at 500 × *g* at room temperature (RT) after 15 min incubation on ice. The pellet was fixed in PME (100 mM PIPES, pH 7.0, 5 mM ethylene glycol tetraacetic acid, 10 mM $MgSO_4$), 2% paraformaldehyde, 0.025% Triton X-100, 100 µM MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester), and 100 µM EGS (ethylene glycol bis[succinimidylsuccinate]) for 30 min at 37°C. Cells were washed with PME and adhered during 15 min to coverslips treated with poly-L-lysine. Cells were washed once with PME and permeabilized during 10 min with PME + 0.1% Triton X-100. Before the treatment with antibodies, cells were washed twice with PME and blocked 30 min with PMEBALG (PME + 1% bovine serum albumin, 0.1% NaN_3, 100 mM lysine, 0.5% coldwater fish skin gelatin [Sigma-Aldrich, St. Louis, MO]). Primary antibodies were diluted with PMEBALG at the working concentrations, and cells were incubated for 2 h at room temperature or overnight at 4°C. After three washes with PME + 0.05% Triton X-100, secondary antibodies were diluted with PMEBALG at the indicated concentrations and used during 1 h at room temperature. After treatment with antibodies, cells were washed three times with PME + 0.05% Triton X-100 and mounted with ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR).

We used the following antibodies for immunofluorescence: anti-HA (monoclonal anti-HA antibody clone HA-7 mouse 1:200), anti-actin (rabbit 1:150) (Paredez *et al*., 2011), anti-tubulin (monoclonal anti-acetylated tubulin antibody clone 6-11B-1 [Sigma], mouse 1:200), anti-centrin (mouse 1:200), and anti-mab414 (mouse 1:1000). Secondary antibodies: Alexa Fluor 488 anti-rabbit (1:200), Alexa Fluor 555 anti-mouse (1:200), Alexa Fluor 594 anti-mouse (1:200), Alexa Fluor-647 anti-mouse (1:200), and Alexa Fluor 555 anti-rabbit (1:200). For experiments in which we needed to use anti-tubulin made in mouse with another antibody made in mouse (anti-HA and anti-centrin), the anti-tubulin antibodies were labeled with the Zenon Alexa Fluor 488 Mouse IgG Labeling Kit according to manufacturer's instructions (Molecular Probes).

Images were acquired using a Deltavision Microscope system with a 100× Olympus objective and a CoolSNAP camera. Deconvolution and projections of images were done with SoftWorx.

Western blot

Cells from the different strains were harvested and lysed in Laemmli SDS sample buffer with Thermo Scientific Halt Protease Inhibitor Cocktail supplemented with EDTA. Samples were resolved in 10% acrylamide gel and transferred to a nonfluorescent polyvinylidene fluoride membrane. The membrane was then probed with anti-HA antibodies (mouse, 1:1500) and anti-actin antibody (rabbit, 1:3000) followed by fluorescent secondary antibodies (IRDye680-linked anti-mouse and IRDye800- linked anti-rabbit, 1:15,000; LI-COR Biosciences) or horseradish peroxidase (HRP) antibodies (1:2500). Western Lightning Plus ECL from Perkin Elmer-Cetus was used for HRP detection. Signals were detected with a Bio-Rad ChemiDoc System imager for the secondary HRP-coupled antibodies, and an Odyssey infrared imager for the fluorescent secondary antibodies.

Protein alignments and sequence tree

Protein sequences were downloaded from the *Giardia* database (http://giardiadb.org/giardiadb) and the National Center for Biotechnology Information database. Sequences were aligned using the software ClustalW in the San Diego Supercomputer Center Biology Workbench (http://workbench.sdsc.edu). CLC Sequence Viewer 6 software was used to give color to the alignments and to create the tree for structural maintenance of chromosomes.

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